

Table 3

Enhancing effect of various attenuated toxins on the antibody production

Attenuated Toxin	Ratio of attenuation (1/4 <sup>th</sup> )	Inoculation Volume (μg/mouse)		Antibody Production HI (2 <sup>nd</sup> )
		Toxin	Influenza HA	
Staphylococcal α Toxin	6.7	0.5	2.0	9.0
	9.1	0.5	2.0	8.6
Pertussis toxin	7.9	1.0	2.0	11.0
	9.3	1.0	2.0	10.8
Diphtheria toxin	7.2	1.0	2.0	10.5
Recombinant of <i>E. coli</i> thermolabile toxin (LT-R7K)	5.8	5.0	2.0	10.9
	9.3	5.0	2.0	11.8
Enteritis vibrio thermostable toxin	8.4	0.5	2.0	9.0
Chorea toxin (control)	0.0	1.0	2.0	11.2
No addition (control)		0.0	2.0	< 4

5 Example 7 - Pharmaceutical preparation containing  
pertussis-diphtheria-tetanus combined vaccine and attenuated cholera  
toxin (nasal drop):

10 Pertussis-diphtheria-tetanus combined vaccine was mixed with  
 an attenuated cholera toxin (having a toxic activity of about 1/100000  
 as compared with that of the natural one) dissolved in PBS and sterilized  
 by filtration. 20 μl of the solution contained the combined vaccine,  
 of which amount corresponded to 50 μg of protein nitrogen, and 5 μg  
 of attenuated cholera toxin. A preservative (0.005% thimerosal) was  
 added to the solution. The resulting mixture was dispensed into  
 15 appropriate containers, which was used as intranasal inoculum  
 comprising pertussis-diphtheria-tetanus combined vaccine and  
 attenuated cholera toxin. The preparation was stored at a temperature  
 of less than 10°C in a cool and dark place.

Vaccine prepared as above containing pertussis-diphtheria-tetanus combined vaccine and attenuated toxin was inoculated to mice of each group. The same amount of the vaccine was further given to each mouse for supplementary inoculation after 4 weeks. After 2 weeks antibody production was evaluated. When vaccine alone was inoculated into mice, the titers (international unit) of anti-pertussis toxin antibody, anti-diphtheria toxin antibody, and anti-tetanus toxin antibody were 2.0 units or lower, 2.0 units or lower, and 1.5 units or lower, respectively. On the other hand, the other group subjected to vaccination with attenuated toxin showed respective titers of 88.6, 61.8, and 75.5 units. The test result indicates that, in the group subjected to vaccination with attenuated toxin, the amount of produced antibodies against the respective antigens are larger than those in mice inoculated with the adjuvant-free vaccines.

Example 8 - Pharmaceutical preparation containing tetanus vaccine and attenuated cholera toxin (percutaneous inoculum):

Tetanus toxoid was prepared as an antigen according to a method described by Kitasato Institute for producing tetanus toxoid ("Textbook of techniques for vaccine production," Kitasato Institute, 1986). Cholera toxin and attenuated cholera toxin (having a toxic activity of about 1/2500 of the natural one) were prepared by the method described in Examples 1 and 2. The two were used as the adjuvants in the test. A tetanus toxoid solution (400 Lf/ml) was mixed with a solution of either cholera toxin (4 mg/ml) or attenuated cholera toxin (4 mg/ml) at various volume ratios to give vaccine preparations (1 ml). After preparation, vaccine was immediately soaked into the cloth base (lint, about 2 cm x 2 cm) and then used for immunization. Guinea pigs (5 in each group) were immunized by the following manner. The back of each guinea pig was shaved with a hair clipper, and then some epilation cream was spread on the back. After 30 minutes, the back of the guinea pig was washed. On the following day, cloth base on which the sample had been spread was patched on the back. The cloth was fixed with an adhesive tape. The mice were bred in the usual manner. The cloth was changed with a fresh one once a week, four times in

total. The first blood collection was carried out on the fourth week after the first patch was given. Six weeks after the primary immunization, the fifth was patched (booster), and then one week after that (seven weeks after the primary immunization), the second blood collection was performed.

A tetanus antibody assay kit "KAKETSUKEN" was used for assaying antibody titer in the blood. The assay result for the titer of antibody against tetanus toxin is shown in Table 4. After seven weeks, the antibody titer was found to be higher in the group subjected to vaccine inoculation with attenuated cholera toxin than that observed in the group subjected to vaccine inoculation without attenuated cholera toxin (control).

Table 4

Test group No.	Tetanus Toxoid (Lf/cloth base)	Toxin (mg/cloth base)	Antibody titer in blood (international unit/mL)	
			After 4 weeks	After 7 weeks
1	100	Attenuated cholera toxin 1.0	3.6	12.3
2	100	Attenuated cholera toxin 0.1	2.6	9.2
3	100	Natural cholera toxin 1.0	4.1	25.6
4	100	Natural cholera toxin 0.1	2.6	14.3
5	100	No addition	2.0 or less	3.1

#### Example 9 - Hepatitis B vaccine-attenuated cholera toxin (injection):

Hepatitis B vaccine was combined with a fraction containing attenuated cholera toxin (having a  $\frac{1}{2}$  toxic activity reduced to at least  $1/10^6$  as compared with that of the natural one) that had been dissolved in PBS and sterilized by filtration. 1 ml of the solution contained HBs antigen, of which amount corresponded to 40  $\mu$ g of protein, and 10  $\mu$ g of the attenuated cholera toxin. A preservative (0.01%